A Simple Radiometric *In Vitro* Assay for Acetylcholinesterase Inhibitors

TOMAS R. GUILARTE, H. DONALD BURNS *x, ROBERT F. DANNALS, and HENRY N. WAGNER, JR.

Received August 10, 1981, from the *Division of Radiation Health Sciences, Department of Environmental Health Sciences, The Johns Hopkins Medical Institutions, Baltimore, MD 21205.* Accepted for publication February 24, 1982. * Present address: Johns Hopkins University, Baltimore, MD 21211.

Abstract □ A radiometric method for screening acetylcholinesterase inhibitors has been described. The method is based on the production of [^{14}C]carbon dioxide from the hydrolysis of acetylcholine. The inhibitory concentration at 50% (IC_{50}) values for several known acetylcholinesterase inhibitors were in agreement with literature values. The new radiometric method is simple, inexpensive, and has the potential for automation.

Keyphrases □ Acetylcholinesterase inhibitors—a simple radiometric *in vitro* assay □ Radiometric method—*in vitro* assay for acetylcholinesterase inhibitors

Acetylcholinesterase activity and the effect of its inhibitors is measured by the rate of hydrolysis of acetylcholine, its natural substrate (1). The rate of acetylcholine hydrolysis can be measured in a variety of ways; the separation and quantitation of [^{14}C]acetic acid from [^{14}C]-acetylcholine is currently the most commonly used method (2–5). However, this method although sensitive and specific, requires some manual manipulation (*e.g.*, extraction of [^{14}C]acetic acid) in measuring acetylcholinesterase activity or in screening compounds exhibiting an inhibitory effect.

In the present report, an alternative radiometric method that is simple, fast, and has the potential for automation is presented.

EXPERIMENTAL

Materials and Methods—Electric eel (1200 U/ml solid) acetylcholinesterase[1] (acetylcholine acetylhydrolase, EC 3.1.1.7), acetylcholine chloride, neostigmine bromide, physostigmine bromide, and decamethonium bromide were obtained from a commercial source[2]. Phenyltrimethylammonium iodide was synthesized by methylation of *N,N*-dimethylaniline with iodomethane and characterized by melting point, elemental analysis, and IR and PMR spectroscopy. *d*-Tubocurarine chloride[3] and edrophonium chloride[4] were obtained from commercial pharmaceutical sources. [^{14}C]Sodium bicarbonate[5] with a specific activity of 56 mCi/mmole was diluted with deionized water to a specific concentration of 1 μCi/0.1 ml. All other reagents were of reagent chemical grade and commercially available. Twenty-milliliter assay vials[6], aluminum seals[7], and rubber liners[8] were obtained from commercial sources. The electric eel acetylcholinesterase was diluted in 5 m*M* $NaHCO_3$ buffer (pH 8.4) to yield a 0.1 U/μl concentration. This enzyme preparation was stable for several days if stored at −20°. Acetylcholine chloride and the various inhibitors were diluted with 5 m*M* bicarbonate buffer (pH 8.4) prior to assay.

Effect of Acetylcholine Concentration on Acetylcholinesterase Activity—The effect of acetylcholine on acetylcholinesterase activity was studied by varying the acetylcholine concentration, as all other variables were fixed. Acetylcholine standard solutions (4 and 15 m*M*) were made with 5 m*M* sodium bicarbonate buffer (pH 8.4). To 20-ml assay vials, 0.25, 0.5, 1, 0, 2.0, and 3.0 ml of the 4 m*M* acetylcholine standard solution and 1.0, 2.0, 3.0, 4.0, and 5.0 ml of the 15 m*M* acetylcholine standard solution were added. The volume was brought to 5 ml with 5 m*M* sodium bicarbonate (pH 8.4). The final pH of vial contents was 8.0. This gave a 1–75 μmole of acetylcholine/vial range. All vials were capped with aluminum seals, which were fitted with rubber liners, and chilled in ice after which 1 μCi (0.1 ml) of [^{14}C]sodium bicarbonate and 10 μliters of acetylcholinesterase (1 U) were injected. Incubation was carried out at 37° for 30 min. After incubation vials were chilled in ice water prior to measuring [^{14}C]carbon dioxide production. Figure 1 represents the effect of increasing concentration of acetylcholine on acetylcholinesterase activity.

An acetylcholinesterase activity *versus* time curve was obtained to check for linearity. To four 20-ml assay vials, 2 ml of a 30 m*M* acetylcholine solution was added and volume made up to 5 ml with 5 m*M* sodium bicarbonate buffer (pH 8.4). One unit of actylcholinesterase and 1 μCi of [^{14}C]sodium bicarbonate were used. Test vials were incubated at 37° for 15-min intervals after which they were chilled in ice and the [^{14}C]carbon dioxide was measured (Fig. 2).

***In Vitro* Inhibition of Acetylcholinesterase**—To screen each of the known acetylcholinesterase inhibitors, the following experiment was performed.

Control Vials—To 2 ml of a 30 m*M* acetylcholine chloride solution, 3 ml of a 5 m*M* bicarbonate buffer (pH 8.4) was added to make a volume of 5 ml.

Test Vials—To test for the inhibitory effect of the various compounds, a series of vials containing the same amount of acetylcholine as the controls with varying concentrations of inhibitor were prepared and the volume made up to 5 ml with 5 m*M* bicarbonate buffer (pH 8.4). All vials

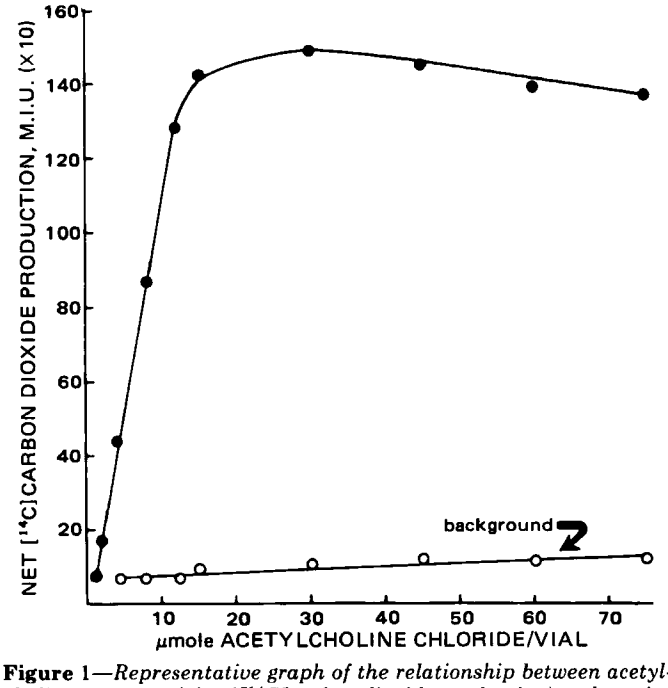


Figure 1—*Representative graph of the relationship between acetylcholinesterase activity ([^{14}C]carbon dioxide production) and varying concentrations of acetylcholine. One microcurie of [^{14}C]sodium bicarbonate and 1 U of electric eel acetylcholinesterase were used. Incubation was done at 37° for 30 min.*

[1] Acetycholinesterase type V-S, Sigma Chemical Co., St. Louis, Mo.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Squibb, Princeton, N.J.
[4] Roche, Belvidere, N.J.
[5] Amersham/Searle, Arlington Heights, Ill.
[6] Arthur H. Thomas, Philadelphia, Pa.
[7] Wheaton, Millville, N.J.
[8] Johnston Laboratories, Cockeysville, Md.

0022-3549/83/0100-0090$01.00/0
© 1983, American Pharmaceutical Association

Table I—Inhibitory Potency of Known Acetylcholinesterase Inhibitors as Ranked by the Radiometric Method

Compound	IC_{50} Value	
	Radiometric, *M*	Literature, *M*
Phenyltrimethylammonium Iodide	2.7×10^{-3}	4.4×10^{-4} (9)
d-Tubocurarine Chloride	1.1×10^{-4}	9.0×10^{-4} (10)
Decamethonium Bromide	3.6×10^{-5}	2.5×10^{-5} (11)
Neostigmine Bromide	6.6×10^{-6}	3.0×10^{-6} (12)
Edrophonium Chloride	1.9×10^{-6}	1.3×10^{-5} (13)
Physostigmine Chloride	2.5×10^{-6}	10^{-6}–10^{-8} (9)

were capped with aluminum seals, which were fitted with rubber liners, and chilled in ice. One microcurie of ^{14}C-labeled sodium bicarbonate followed by 1 U (10 μl) of electric eel acetylcholinesterase were injected into each vial. Appropriate blanks (*i.e.*, background) to account for nonenzymatic hydrolysis of acetylcholine were prepared. The background vials contained all ingredients as test and control vials with the exception that no enzyme was added, thus, measuring nonenzymatically hydrolyzed acetylcholine. All vials were incubated at 37° for 30 min. The concentration of inhibitor which reduced the control value by 50% was defined as inhibitory concentration 50% (IC_{50}). This value was determined from the net [^{14}C]carbon dioxide production *versus* inhibitor concentration curve (Fig. 3).

Measurement of [^{14}C]Carbon Dioxide Produced—The amount of [^{14}C]carbon dioxide generated was quantified using a bacteriological detection system[9] which contained an ionization chamber. [^{14}C]Carbon dioxide activity was expressed as metabolic index units (miu.), where 100 miu. equals 0.031 μCi of [^{14}C]carbon dioxide. This instrument is widely used in clinical laboratories as well as in industry. Technical details have been described elsewhere (6, 7).

RESULTS AND DISCUSSION

A new and simple radiometric method was developed to screen potential acetylcholinesterase inhibitors. This technique is based on the hydrolysis of acetylcholine by acetylcholinesterase to acetic acid and choline. The acetic acid formed from the enzymatic hydrolysis of acetylcholine reacts with [^{14}C]sodium bicarbonate to generate [^{14}C]carbon dioxide, which is measured using the ionization chamber system. The

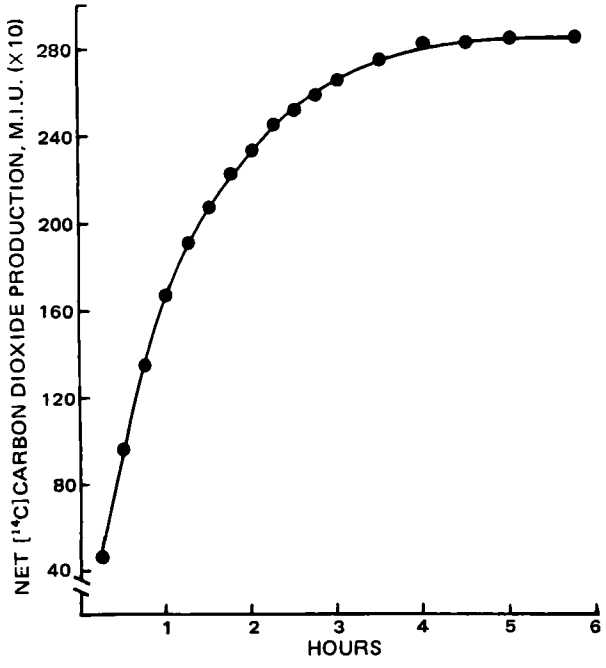


Figure 2—*Representative graph of electric eel acetylcholinesterase activity (1 U) versus time. One microcurie of [^{14}C]sodium bicarbonate and a 30 mM acetylcholine solution was used as substrate. Test vials were incubated for 15-min intervals at 37° after which [^{14}C]carbon dioxide production was quantified.*

[9] Bactec R301, Johnston Laboratories, Cockeysville, Md.

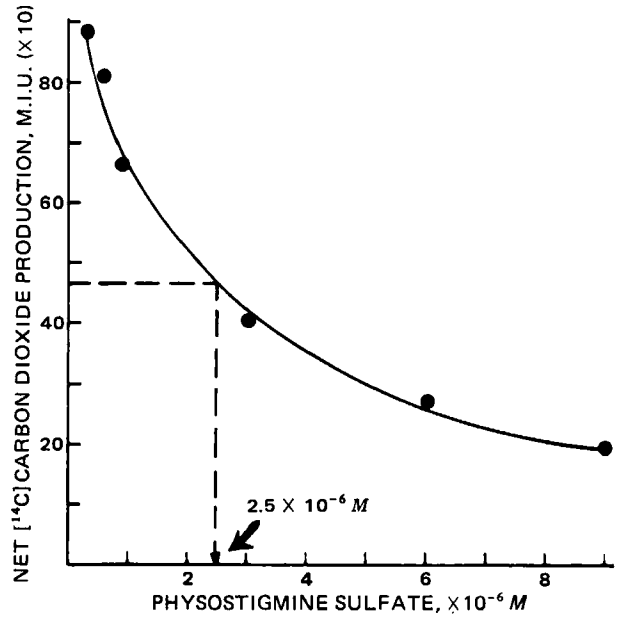


Figure 3—*Effect of increasing concentration of physostigmine sulfate on the activity of 1 U of electric eel acetylcholinesterase. IC_{50} represents the concentration of inhibitor which reduces the production of [^{14}C]-carbon dioxide by 50% of control vial containing no inhibitor. Incubation time was 30 min at 37°. (Control = 928; IC_{50} = 928/2 = 464.)*

[^{14}C]carbon dioxide evolved is proportional to the amount of acetylcholine hydrolyzed (Fig. 1).

The effect of increasing concentrations of acetylcholine on acetylcholinesterase activity was studied. Figure 1 indicates that for 30-min incubation at 37° there was a linear response of acetylcholinesterase activity from 1 to 15 μmoles of acetylcholine/vial followed by saturation at 3×10^{-3} *M* acetylcholine concentration and an actual decrease in activity as the substrate concentration increases. The decrease on acetylcholinesterase activity is a result of substrate inhibition (8). An acetylcholinesterase activity *versus* time curve was obtained to determine the incubation time of test vials (Fig. 2).

Preliminary studies with known acetylcholinesterase inhibitors have shown that the new radiometric method can be used to rank such compounds according to their inhibitory potency on acetylcholinesterase (Table I). Even though comparison of absolute IC_{50} values cannot be made between the described method and those obtained by other methods due to different experimental conditions, the results presented are in general agreement with those reported in the literature (Table I).

The new radiometric method is simple, fast, and inexpensive and has the potential for automation. Many samples can be prepared and analyzed in a few hours. It can be applied as a rapid test in the screening of such compounds as pesticides and chemicals of medical interest.

The method is not only applicable for assaying acetylcholinesterase activity and the effect of its inhibitors, but it can be extended to other enzyme systems in which a proton is generated as a result of the enzymatic action.

REFERENCES

(1) A. R. Main, in "Biology of Cholinergic Function," A. M. Goldberg and I. Hanin Eds., Raven, New York, N.Y., 1976, pp. 269–353.

(2) F. Fonnum, *Biochem. J.*, **115**, 465 (1969).

(3) M. W. McCaman, L. R. Tomey, and R. E. McCaman, *Life Sci.*, **7**, 233 (1968).

(4) L. T. Potter, *J. Pharm. Exp. Ther.*, **156**, 500 (1967).

(5) D. J. Reed, K. Goto, and C. H. Wang, *Anal. Biochem.*, **16**, 59 (1966).

(6) F. H. DeLand, in "Nuclear Medicine In-Vitro," P. Rothfeld, Ed., J. P. Lippincott, Philadelphia, Pa., 1974, pp. 384–403.

(7) J. R. Waters and A. A. Zwarun, in "Developments in Industrial Microbiology," vol. XIV, American Institute of Biological Sciences, Washington, D.C., 1973, pp. 80–89.

(8) K. B. Augustinsson, *Arch. Biochem.*, **23**, 111 (1949).

(9) J. P. Long, in "Handbuch der Experimenteelen Pharmakologie," Suppl. 15, Springer-Verlag, Berlin, West Germany, 1963, pp. 377–384.

(10) F. Bergmann, F. B. Wilson, and D. Nachmansohn, *Biochem. Biophys. Acta*, **6**, 217 (1950).
(11) F. Bergmann, I. B. Wilson, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 693 (1950).
(12) C. M. Smith, S. L. Cohen, E. W. Pelikan, and K. R. Unna, *J. Pharmacol.*, **105**, 391 (1952).
(13) H. L. Cohen and K. R. Unna, *ibid.*, **103**, 340 (1951).

ACKNOWLEDGMENTS

This work was supported by the U.S. Public Health Service Research Grant GM-10548.
The authors thank Dr. Alan Goldberg from the Division of Toxicology, Department of Environmental Health Sciences for his encouragement and advice.

Microbial Metabolism Studies on the Major Microbial and Mammalian Metabolite of Primaquine

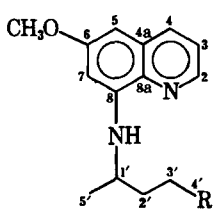
CHARLES D. HUFFORD *x, ALICE M. CLARK *, IRIS N. QUINONES *, JOHN K. BAKER ‡, and J. D. McCHESNEY *

Received December 12, 1981, from the **Department of Pharmacognosy* and the ‡*Department of Medicinal Chemistry, School of Pharmacy, the University of Mississippi, University MS 38677.* Accepted for publication February 26, 1982.

Abstract □ The microbial metabolism of 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (II), a known microbial and mammalian metabolite of the antimalarial drug primaquine (I), was investigated using selected organisms. *Streptomyces rimosus* produced a single metabolite that was identified as an amide derivative of II (V) by spectroscopic methods. The amide (V) was synthesized by ammonia treatment of the methyl ester (IV). The lactam derivative (VI) was also prepared by treatment of II with *N*-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline.

Keyphrases □ Primaquine—microbial studies on the major microbial and mammalian metabolite, high-performance liquid chromatography □ High-performance liquid chromatography—microbial metabolism studies on the major microbial and mammalian metabolite of primaquine

The utilization of microorganisms as models for studying mammalian metabolism is a concept that has been advocated as an aid for future metabolic studies (1). Recent studies with phencyclidine (2) and imipramine (3) have shown that microorganisms do produce the same metabolites as mammalian systems. Primaquine (I), an antimalarial drug, has also been subjected to a microbial metabolic study since little or no information has been reported regarding its mammalian metabolism (4). The two major microbial metabolites identified were the carboxylic acid derivative (II) and the *N*-acetyl derivative of the primary amine (III). In parallel studies, the mammalian metabolism of I using rats has been studied, and II has been identified as the major mammalian metabolite as well (5). The tissue distribution of I has been studied, and even though substantial evidence indicated that I was rapidly metabolized by the rat, no mammalian metabolites were identified (6). Having microbial metabolites as reference standards for conducting mammalian metabolic studies can be useful. The purpose of this investigation was to study further the biotransformations of the major mammalian metabolite (II) of primaquine using microbial systems.



I: R = $-CH_2NH_2$
II: R = $-CO_2H$
III: R = $-CH_2NHCOCH_3$
IV: R = $-CO_2CH_3$
V: R = $-CONH_2$

RESULTS AND DISCUSSION

A total of 60 microorganisms typical of those used previously (3, 7) were used in the screening of 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (II) for microbial metabolites. Of these, *Streptomyces rimosus*[1] was chosen for preparative scale fermentations, since TLC indicated complete conversion in 3 days to one major metabolite. Other microorganisms showing this same metabolite as determined by TLC were *Streptomyces flocculus*[2], *Polyporus sanguineus*[3], and *Fusarium oxysporum* f. sp. *cepae*[4] although large amounts of II were also present even after 7–10 days.

Incubation of 300 mg of II with submerged cultures of *S. rimosus* for 4 days and extraction of the entire culture with ethyl acetate resulted in 700 mg of ethyl acetate solubles. Chromatography of a 400-mg sample of this residue using preparative layer silica gel and alumina plates resulted in the isolation of 18 mg of the pure metabolite by TLC and HPLC (overall yield, 12%).

The ^{1}H-NMR spectrum of the metabolite showed the aromatic protons to have nearly the same δ positions and J values as reported for II (4). The spectrum also confirmed that the methoxyl group and the secondary methyl were present. This suggested that the biotransformation had occurred in the side chain. The mass spectrum was consistent for $C_{15}H_{19}O_2N_3$ (M^+ 273, 10%) and showed significant fragments at m/z 215 (51%) and 201 (100%), further confirming that the aromatic nucleus had not been transformed (4). The IR spectrum indicated absorption at 3400, 3510, and 1760 cm^{-1}. The ^{13}C-NMR spectral data (Table I) was nearly identical to that of the methyl ester of II (IV). The collective spectroscopic data suggested the microbial metabolite was an amide derivative represented by V. This was confirmed by treating IV with methanol saturated with ammonia in a sealed tube at 140°. The product of this reaction was identical in all respects to the metabolite (V). No evidence of the lactam derivative (VI) was noted in this reaction, although VI was noted as a product of heating IV in the gas chromatograph (5). Attempts to prepare

[1] American Type Culture Collection (ATCC), 23955, Rockville, Md.
[2] ATCC 25453.
[3] ATCC 14622.
[4] ATCC 11711.

0022-3549/83/0100-0092$01.00/0
© 1983, American Pharmaceutical Association